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# Potent block of potassium currents in rat isolated sympathetic neurones by the uncharged form of amitriptyline and related tricyclic compounds

Julian R.A. Wooltorton & [1]Alistair Mathie

Department of Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF

1 The block of $K^+$ currents by amitriptyline and the related tricyclic compounds cyproheptadine and dizocilpine was studied in dissociated rat sympathetic neurones by whole-cell voltage-clamp recording.

2 Cyproheptadine (30 $\mu$M) inhibited the delayed-rectifier current ($K_V$) by 92% and the transient current ($K_A$) by 43%. For inhibition of $K_V$, cyproheptadine had a $K_D$ of 2.2 $\mu$M. Dizocilpine (30 $\mu$M) inhibited $K_V$ by 26% and $K_A$ by 22%. The stereoisomers of dizocilpine were equally potent at blocking $K_V$ and $K_A$.

3 Amitriptyline, a weak base, was significantly more effective in blocking $K_V$ at pH 9.4 ($K_D = 0.46$ $\mu$M) where the ratio of charged to uncharged drug was 50:50 compared with pH 7.4 ($K_D = 11.9$ $\mu$M) where the ratio was 99:1.

4 N-methylamitriptyline (10 $\mu$M), the permanently charged analogue of amitriptyline, inhibited $K_V$ by only 2% whereas in the same cells amitriptyline (10 $\mu$M) inhibited $K_V$ by 36%.

5 Neither amitriptyline nor N-methylamitriptyline had a detectable effect on $K_V$ when added to the intracellular solution.

6 It is concluded that the uncharged form of amitriptyline is approximately one hundred times more potent in blocking $K_V$ than the charged form. However, this does not seem to be due to uncharged amitriptyline having better access to an intracellular binding site.

**Keywords:** Amitriptyline; N-methylamitriptyline; cyproheptadine; dizocilpine; potassium currents; rat sympathetic neurones; whole-cell voltage-clamp; pH

## Introduction

Previously, we have shown that two voltage-gated potassium ($K^+$) currents in rat superior cervical ganglion (SCG) neurones, the delayed-rectifier current $K_V$ and the transient current $K_A$, can be inhibited by certain tricyclic antidepressants (e.g. amitriptyline and imipramine) and other structurally related compounds such as chlorpromazine (Wooltorton & Mathie, 1993). Potassium channels play an important role in the regulation of neuronal membrane potential and neuronal excitability (Cook & Quast, 1990; Halliwell, 1990), therefore compounds like these which inhibit $K^+$ currents will have a number of actions on neurones which may be either therapeutic or toxic.

This study was divided into two parts. In the first series of experiments, we have extended our observations to two further tricyclic compounds; cyproheptadine and dizocilpine. Cyproheptadine is an antagonist at 5-hydroxytryptamine and histamine ($H_1$) receptors used clinically in certain allergies such as hay-fever and occasionally in migraine (Laurence & Bennett, 1992); while dizocilpine (MK-801) is a non-competitive NMDA receptor antagonist with anticonvulsant properties (Wong *et al.*, 1986).

In the second series of experiments, we have studied in more detail the mechanism of action of these tricyclic compounds in inhibiting $K_V$. In particular, since all of the active compounds are weak bases, we have determined whether the protonated (charged) or unprotonated (uncharged) form of these molecules is more important for their inhibitory action by varying the pH of the extracellular recording solution and by using a permanently charged analogue of amitriptyline, N-methylamitriptyline.

[1] Author for correspondence.

Some of the results in this study have been published in abstract form (Wooltorton & Mathie, 1994; 1995).

## Methods

### *Cell dissociation*

Neurones were dissociated from rat superior cervical ganglia (SCGs) by a method modified from one described previously (Beech *et al.*, 1991; Bernheim *et al.*, 1991). Sprague-Dawley rats of either sex (age 7-8 days) were killed by decapitation. The SCGs were removed and placed at 37°C in a modified Hank's solution containing 20 iu $ml^{-1}$ papain for 20 min. The ganglia were then incubated in a mixture of 400 iu $ml^{-1}$ collagenase (Type I) and 16 mg $ml^{-1}$ dispase (Grade II) for 45 min and triturated every 15 min. Cells were then centrifuged and resuspended in Leibovitz L-15 medium. Isolated neurones were kept at 4°C and used for recording within 10 h.

### *Solutions*

The external solution (in mM) was NaCl 150, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 10, glucose 8, TTX 0.0005 and the pH was adjusted with NaOH as required (physiological pH = 7.4). The internal solution (in mM) was: KCl 145, $MgCl_2$ 5, HEPES 5, BAPTA 10 and the pH was adjusted to 7.4 with KOH.

Stock solutions of all compounds were made up in distilled water except for cyproheptadine, which was made up in 50% ethanol, and were kept at -20°C until required. They were then added to the external solutions at suitable concentrations shortly before the experiment. Solutions were applied to the cells by continuous perfusion of the chamber

during recording. The perfusion rate was 4–5 ml $min^{-1}$ and complete exchange of the bath solution occurred within 20–40 s.

Papain, collagenase, cyproheptadine, tacrine and amitriptyline were obtained from Sigma, (+)- and (−)-dizocilpine from Semat Technical (RBI), dispase from Boehringer Mannheim and Leibovitz L-15 from Life Technologies (Gibco). N-methylamitriptyline was a generous gift from Dr F. J. Ehlert.

### *Current recording and analysis*

Currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) at 20–23°C with an Axopatch 1D amplifier. Patch pipettes had resistances of between 2 and 5 MΩ. During whole-cell recording, series resistance was 14.3 ± 0.8 MΩ and the whole-cell capacitance was 14.1 ± 0.8 pF ($n$ = 60). Currents were not leak-subtracted.

*Voltage protocols* *Protocol (1):* To measure $K_V$ and $K_A$, cells were held at −30 mV and a conditioning pulse to −120 mV was applied for 500 ms, once every 5 s, before a test pulse of 400 ms to 0 mV. The peak current, measured within 10 ms of the step to 0 mV, has been shown to be predominantly $K_A$, while the sustained current measured as an average over 100 ms, 297 ms following the step was predominantly $K_V$ (see Wooltorton & Mathie, 1993).

*Protocol (2):* To measure $K_V$ in isolation, cells were held at −70 mV and stepped once every 6 s to −50 mV for 30 ms to inactivate any residual $K_A$, then depolarized to a test potential of +10 mV for 150 ms before stepping back first to −50 mV for 30 ms then to the holding potential. $K_V$ was measured as the average current over 25 ms, 116.5 ms following the step to +10 mV.

*Protocol (3):* To study the steady state inactivation of $K_A$ and $K_V$, cells were held at −70 mV and stepped once every 5 s to potentials between −120 mV and +30 mV in 10 mV increments for 2 s, and depolarized to +50 mV for 90 ms. $K_A$ was measured at the time where peak current after the depolarizing step from − 120 mV occurred. $K_V$ was measured as the mean current over 25 ms, 62.5 ms after the depolarizing step.

Currents were low-pass filtered at 5 kHz, digitized at 0.5–5 kHz and recorded and analysed using an IBM-compatible PC, pClamp version 5.5 or version 5.7 with a TL-1 Labmaster or a Digidata 1200 interface (Axon Instruments) and Excel version 4.0 (Microsoft). Statistical tests were carried out using a Student's $t$ test. $P$ values of less than 0.05 were considered significant. All data are expressed as mean ± s.e.mean and $n$ is the number of cells.

The concentration of amitriptyline and N-methylamitriptyline entering a cell from the recording pipette was calculated as described in Stansfeld & Mathie (1993) (see also Beech *et al.*, 1991; Pusch & Neher, 1988). Briefly, previous studies have shown that BAPTA (mol. wt. 476.4) diffused into sympathetic neurones (Rs = 3.5 MΩ; Cs = 36 pF) with a time constant, $\tau_B$ of 362 s (Beech *et al.*, 1991; Stansfeld & Mathie, 1993). Thus BAPTA diffusion into any similar sized neurone of interest can be calculated from equation 1:

$$\tau_{Bu} = (RuCu^{3/2}/RsCs^{3/2})\tau_B \quad [1]$$

where $\tau_B$ = 362s, Rs = 3.5 MΩ, Cs = 36 pF, Ru and Cu are the series resistance and whole-cell capacitance of the neurone of interest and $\tau_{Bu}$ is the time constant of BAPTA entry into this neurone. Once this is known, the time constant for entry of any compound (of reasonably similar molecular weight) can be calculated from equation 2. So, for example, for N-methylamitriptyline:

$$\tau_{amiM} = \tau_{Bu}(M_{amiM}/M_{BAPTA})^{1/3} \quad [2]$$

where $\tau_{amiM}$ is the time constant for N-methylamitriptyline and $M_{amiM}$ and $M_{BAPTA}$ are the two compounds respective molecular weights (476.4 and 419.3). The concentration of N-methylamitriptyline inside the cell is then known for any time following breakthrough and from equation 3 and the values obtained from the amitriptyline dose-response curve (see Results), the degree of block of $K_V$ at any time following breakthrough can be calculated if it is assumed that the charged form of amitriptyline blocks from inside the cell. This is shown for amitriptyline in Figure 10a and N-methylamitriptyline in Figure 10b.

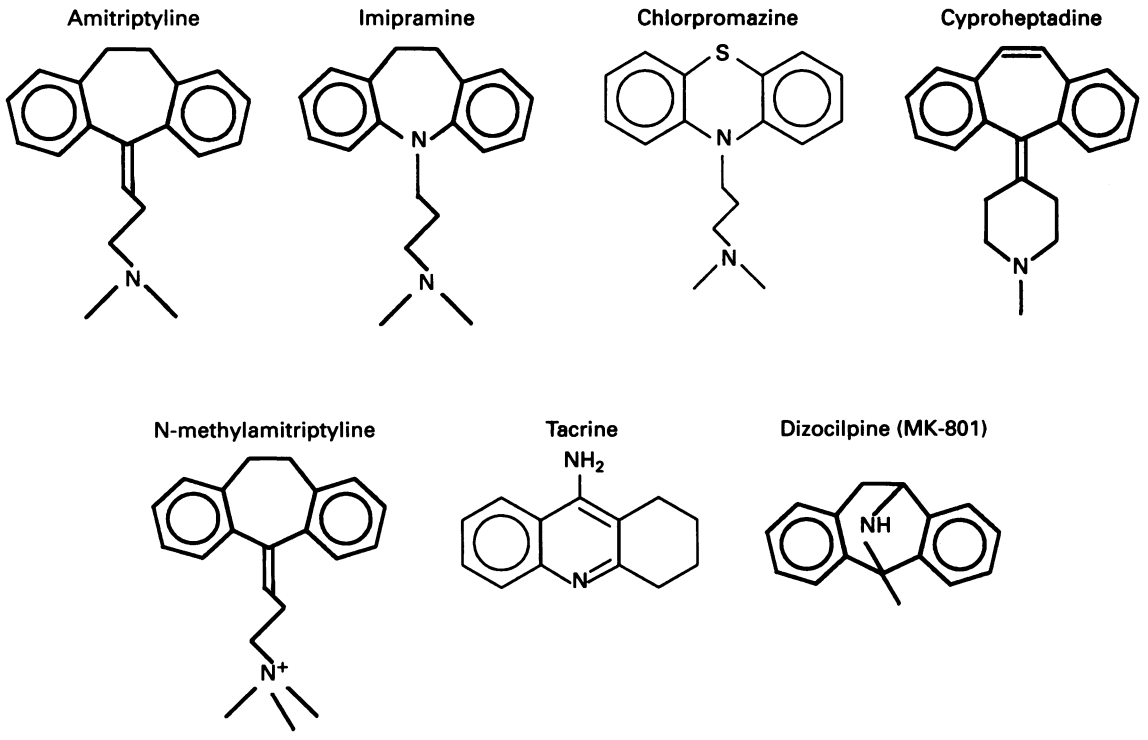


**Figure 1** Chemical structures of the tricyclic compounds investigated.

*Structures of tricyclic compounds*

The structures of the compounds studied are shown in Figure 1. They all possess a tricyclic region. Imipramine, amitriptyline, chlorpromazine and cyproheptadine each has a tertiary amine group on the central side chain substituent, positioned three carbon atoms from the cyclic structure, which differs slightly for each drug. In contrast, tacrine and dizocilpine have no such side chain. N-methylamitriptyline is a permanently charged analogue of amitriptyline, having the same structure except for a quaternary amine group in place of the tertiary amine group.

## Results

*Block of $K_A$ and $K_V$ by cyproheptadine and dizocilpine*

Both the transient and sustained potassium currents ($K_A$ and $K_V$) can be evoked by voltage protocol 1 (see Methods) and are illustrated in Figure 2a. The control peak current (at 0 mV) for the cells used in this study was 2417 ± 191 pA ($n$ = 60) while the control sustained current was 621 ± 42 pA.

Figure 2 also illustrates the effect of various concentrations of cyproheptadine on $K_A$ and $K_V$. The inhibition of both currents was concentration-dependent and there appeared to be selectivity for $K_V$ over $K_A$ (Figure 2b). For example, 30 μM cyproheptadine produced an inhibition of $K_V$ of 92.2 ± 1.6% ($n$ = 6) but inhibited $K_A$ by only 43.3 ± 2.7% (data not shown). Figure 2a also shows the hyperpolarization-activated chloride current of these neurones during the prepulse to −120 mV (Clark & Mathie, 1995). It is of interest that cyproheptadine causes a slight enhancement of this current.

Figure 3 shows the effect of the two stereoisomers of dizocilpine (30 μM) on $K_A$ and $K_V$. Both produced similar inhibitions of $K_A$ and $K_V$. In 4 cells, (+)-dizocilpine (the potent NMDA antagonist) inhibited $K_V$ by 26.0 ± 3.7% and inhibited $K_A$ by 22.0 ± 3.6%; the stereoisomer (−)-dizocilpine inhibited $K_V$ by 23.1 ± 4.8% and $K_A$ by 16.6 ± 3.3%. Inhibition of either current by (+)-dizocilpine was not significantly different from

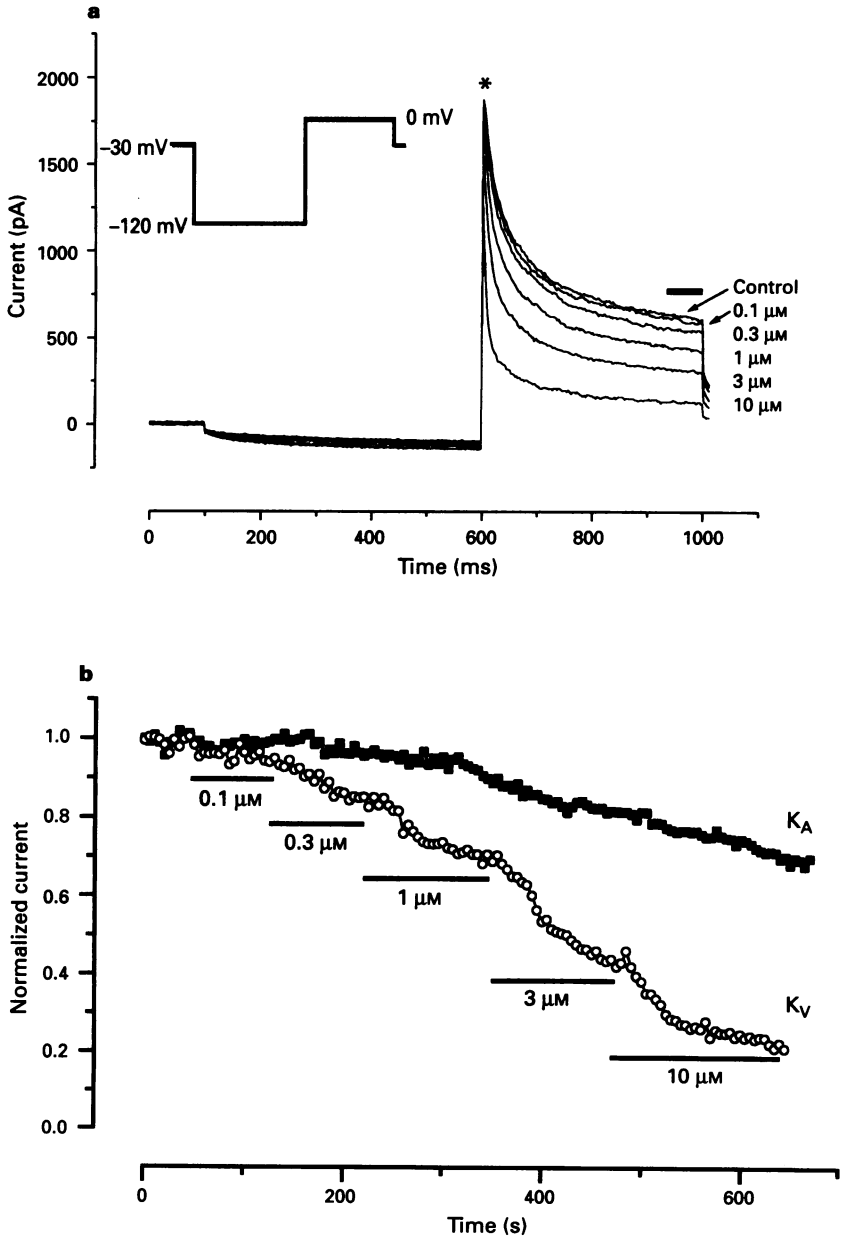


**Figure 2** The effects of various concentrations of cyproheptadine on $K_A$ and $K_V$ are shown. The currents were activated using voltage protocol (1). In (a), the average of 3 traces in control solution and at each concentration are shown for clarity. In (b) (■) represent $K_A$, measured at * in (a); (○) represent $K_V$ measured as indicated by the bar in (a). The bars in (b) indicate the time of application of cyproheptadine. Note the apparent selectivity of cyproheptadine for $K_V$ over $K_A$.

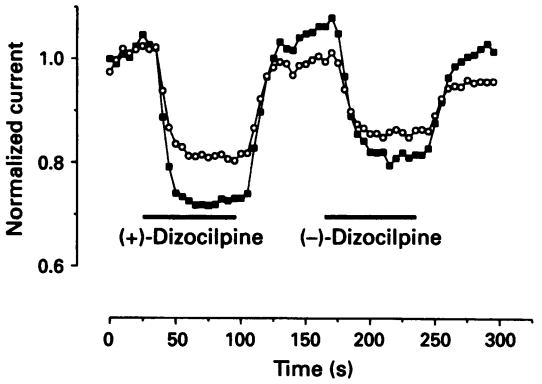


**Figure 3** The effect of 30 $\mu$M (+)- and (−)-dizocilpine on $K_A$ (○) and $K_V$ (■) is shown. In this cell, (+)-dizocilpine inhibited $K_V$ by 28% and $K_A$ by 19%. (−)-Dizocilpine inhibited $K_V$ by 19% and $K_A$ by 17%. Currents were activated using voltage protocol (1).

inhibition by (−)-dizocilpine ($P>0.05$). The non-selectivity of dizocilpine for either current and its lower potency compared with cyproheptadine is consistent with our previous hypothesis that, for block of potassium channels by a family of tricyclic compounds, a central chain, as is present in amitriptyline and cyproheptadine for example, is important for the selectivity of block for $K_V$ over $K_A$ (Wooltorton & Mathie, 1993).

### *Concentration-dependent inhibition of $K_V$ by cyproheptadine and amitriptyline*

The concentration-dependence of inhibition of $K_V$ by cyproheptadine and amitriptyline is illustrated in Figure 4(a) together with concentration-response curves for imipramine and tacrine obtained previously (Wooltorton & Mathie, 1993) for comparison. Data were fitted with the equation:

$$y = \min + ((100\text{-}\min)/(1 + (x/K_D)^s)) \quad [3]$$

where min is the minimum percentage current attainable, and s is the slope factor (Hill coefficient). For cyproheptadine, the $K_D$ was 2.2 ± 0.53 $\mu$M, min was 5.3 ± 3.0% and $s$ was 1.4 ± 0.33. For amitriptyline, the $K_D$ was 11.9 ± 1.2 $\mu$M, min was −1.0 ± 3.3% and s was 0.90 ± 0.05.

Figure 4(b) shows these concentration-response curves plotted as a function of external uncharged drug concentration (and not total concentration). The uncharged concentration was calculated using the equation:

$$\log([U]/[C]) = pH - pK_a \quad [4]$$

where [U] is the concentration of uncharged drug and [C] is the concentration of charged drug in solution. The close superposition of the curves illustrates a good correlation between inhibition of $K_V$ and the uncharged concentration of drug in the external solution. This suggests that there may be a relationship between the $pK_a$ value for each drug and the potency of block of $K_V$.

Table 1 summarizes the inhibitions of $K_V$ by 10 $\mu$M concentrations of various tricyclic compounds. This table shows that there indeed appears to be some correlation between $pK_a$ and inhibition of $K_V$: for a higher $pK_a$ value the inhibition of $K_V$ is reduced and suggests that the charged and uncharged forms of these drugs may have a different potency for blocking $K_V$.

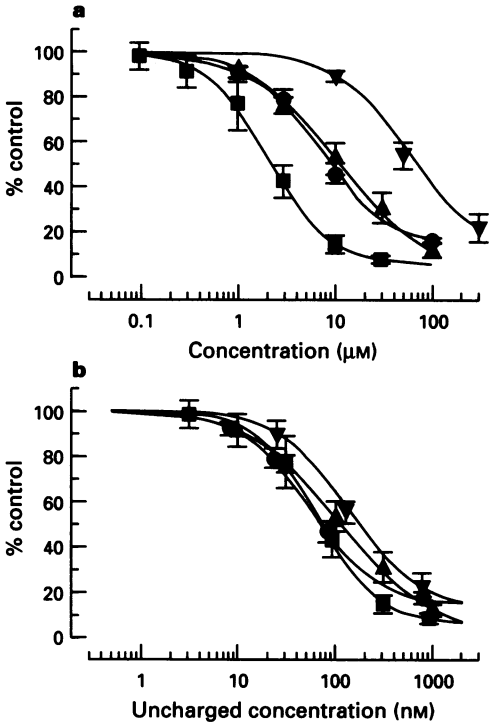


**Figure 4** The concentration-dependent inhibition of $K_V$ by cyproheptadine (■) and amitriptyline (▲) is shown in (a), together with data for imipramine (●) and tacrine (▼) presented previously. Data are fitted using equation [3] in the text. (b) Shows the data from (a) plotted against the concentration of uncharged drug present in the external solution as calculated from equation [4]. Note the close superposition of each curve. Currents were activated using voltage protocol (1).

Experiments were carried out to determine the effect of changing the relative proportion of charged to uncharged drug. In the first set of experiments we looked at the effect of changing external pH on the degree of block produced by amitriptyline (chosen as a representative drug) while in the second set we looked at the effectiveness of the permanently charged analogue of amitriptyline; N-methylamitriptyline. Firstly, however, it was important to measure the effect of alterations of extracellular pH on the potassium currents themselves.

### *Effect of pH on $K_A$ and $K_V$*

Changing pH from 7.4 to 9.4 caused a small but consistent increase in the amplitude of $K_V$ without any obvious effect on the shape of the current and therefore the activation or inactivation rates (see for example Figure 6). This increase in amplitude, also seen for $K_A$, can be explained by relatively small pH dependent shifts in the steady-state activation and inactivation curves for both currents. Figure 5 illustrates the effect of pH on the steady state inactivation of these currents.

**Table 1** Comparison of $pK_a$ values for 5 tricyclic compounds and percentage inhibition of $K_V$ produced by 10 $\mu$M of the same compounds

| *Compound* | *Cyproheptadine* | *Imipramine* | *Chlorpromazine* | *Amitriptyline* | *Tacrine* |
|---|---|---|---|---|---|
| $pK_a$ | 8.9 | 9.5 | 9.3 | 9.4 | 10.0 |
| $K_V$ inhibition by 10 $\mu$M drug | 85.7 ± 3.6 ($n$=6) | 54.0 ± 4.5 ($n$=9) | 53.3 ± 2.3 ($n$=6) | 47.2 ± 2.5 ($n$=13) | 10.2 ± 2.0 ($n$=5) |

Imipramine, chlorpromazine, amitriptyline and tacrine data are from Wooltorton & Mathie (1993).

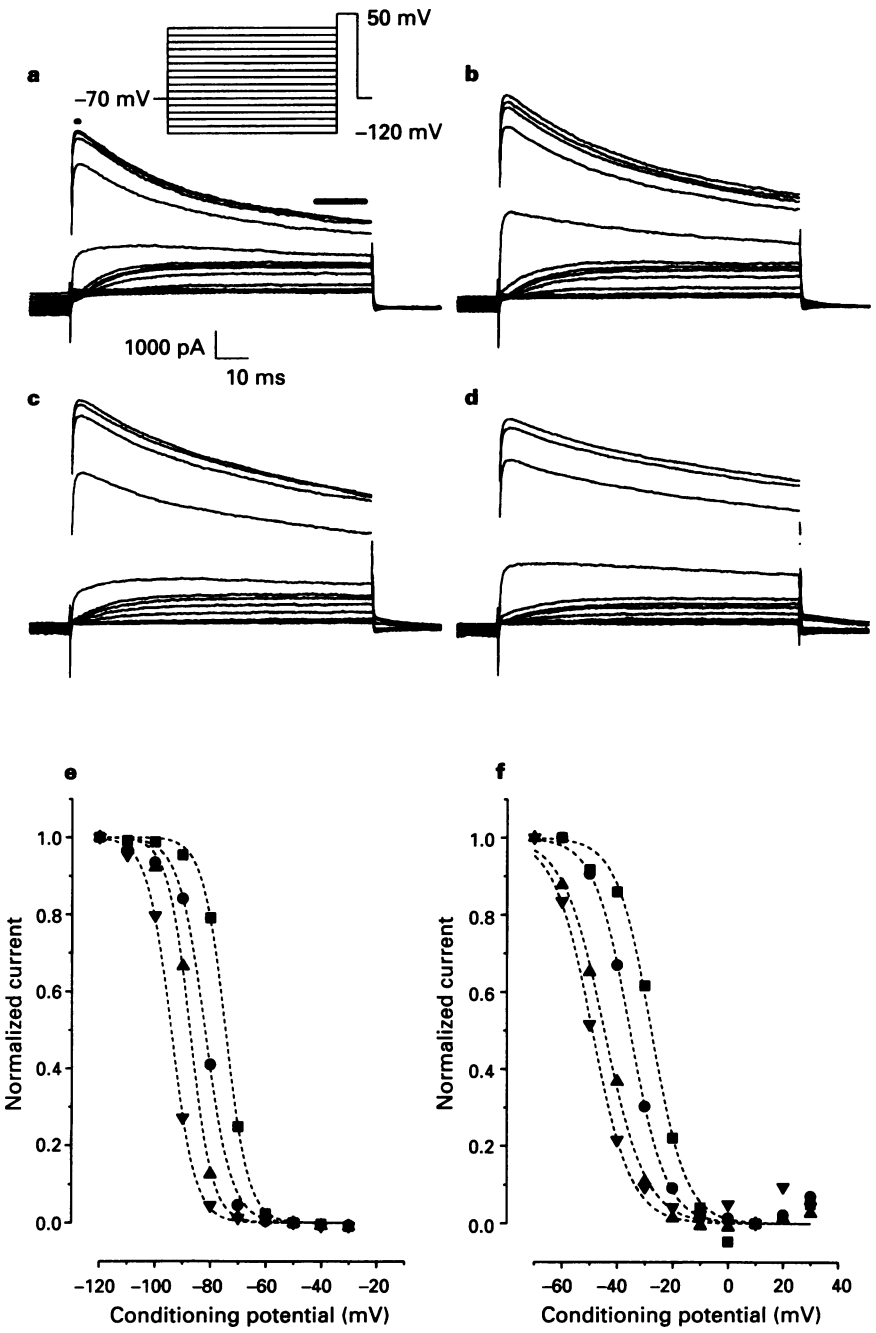


**Figure 5** The effect of pH on the steady state inactivation of $K_A$ and $K_V$. The currents were activated by voltage protocol (3). (a) to (d) Show the current evoked by the depolarizing step to +50 mV from the various conditioning steps at pH 7.4, 7.9, 8.4 and 9.4, respectively. Data for $K_A$ (e) and $K_V$ (f) were fitted with a single Boltzmann function at each pH, (■)=pH 7.4, (●)=pH 7.9, (▲)=pH 8.4 and (▼)=pH 9.4. The dot and bar in (a) show where current amplitudes were measured.

$K_A$ was taken at the time for the peak current evoked by the depolarization from −120 mV and $K_V$ as the average current over 25 ms, 62.5 ms after the depolarization. Currents were normalized to their respective values evoked from the hyperpolarization to −120 mV and fitted by a single Boltzmann function. The $V_{50}$ (the potential at which 50% of the maximal current was inactivated) for steady state inactivation of $K_V$ was −28.1 ± 1.6 mV ($n$=11) at pH 7.4 and −44.7 ± 5.3 mV at pH 9.4. For $K_A$, $V_{50}$ was −79.3 ± 2.2 mV at pH 7.4 and −90.8 ± 4.0 mV at pH 9.4. Thus an increase in pH resulted in a significant shift ($P$<0.05) in the hyperpolarizing direction of the inactivation curves. There was no significant difference in the slope factors for any pH when compared to pH 7.4 ($P$>0.05). Similar hyperpolarizing shifts in the steady-state activation curves were seen for both currents (data not shown).

With the voltage protocols used in this study, the number of channels available for activation will be relatively unaltered by the shift in the steady-state inactivation curve on changing pH from 7.4 to 9.4, while the number of channels activated by steps to 0 mV or +10 mV will be increased, because of the shift in the activation curve, giving the resulting increase in current amplitude.

### *Inhibition of $K_V$ by TEA and amitriptyline at pH 7.4 and 9.4*

Figures 6 and 7 show the effects of 5 mM TEA (a permanently-charged selective blocker of $K_V$) and 10 μM amitriptyline on $K_V$ at physiological (7.4) and elevated (9.4) pHs. At both pHs, 5 mM TEA caused no significant inhibition of $K_A$. However, this concentration of TEA inhibited $K_V$ by 58.5 ± 3.0% ($n$=6) at pH 7.4 and 57.1 ± 6.1% ($n$=5) at pH 9.4. There is no sig-

nificant difference between these values ($P>0.05$). Note that the wash off was very similar at both pHs for TEA (Figure 6b). This suggests that the effect of TEA on potassium channels is not pH-dependent, i.e. the binding of the drug molecule to the channel protein is unaffected by pH.

At pH 7.4, 10 $\mu$M amitriptyline blocked $K_V$ to a similar degree to that seen before (Wooltorton & Mathie, 1993): $47.7\pm5.0\%$ ($n=10$). In contrast to TEA, however, inhibition of this current, in the same 10 cells, was significantly larger at the elevated pH ($79.7\pm5.6\%$) illustrating a distinct pH-dependence for amitriptyline. It can also be seen that the time taken for wash-off was much longer for amitriptyline at the elevated pH (Figure 6c).

### *Concentration-dependent inhibition of $K_V$ by amitriptyline at pH 7.4 and 9.4*

Figure 8 shows a concentration-response curve for amitriptyline blocking $K_V$ at both pH 7.4 and 9.4. Figure 8a illustrates the percentage inhibition of $K_V$ plotted against total drug concentration. The $K_D$ for amitriptyline at pH 7.4 was $11.9\pm1.2$ $\mu$M with minimum attainable current of $-1.0\pm3.3\%$ and slope of $0.90\pm0.05$ as shown in Figure 4. At pH 9.4, amitriptyline was about 25 times more potent with a $K_D$ of $0.46\pm0.08$ $\mu$M, a minimum attainable current of $16.8\pm4.2\%$ and a slope of $0.98\pm0.13$ ($n=5-10$ cells at each concentration). To confirm that this result was not unique to amitriptyline, similar experiments were carried out with cyproheptadine and tacrine. At pH 9.4, 120 nM cyproheptadine inhibited $K_V$ by 67% ($n=2$) while 623 nM tacrine inhibited $K_V$ by $25\pm2\%$ ($n=3$). These drug concentrations would produce no detectable inhibition of $K_V$ at pH 7.4 (see Figure 4); however, they were calculated to contain the same concentration of uncharged drug as 3 $\mu$M cyproheptadine (uncharged concentration, 90 nM) and 50 $\mu$M tacrine (uncharged concentration, 125 nM) at pH 7.4, concentrations which significantly inhibit $K_V$ at this pH ($76\pm7\%$, $n=3$ and $40\pm4\%$, $n=3$, respectively, $P<0.05$). These results illustrate the correlation

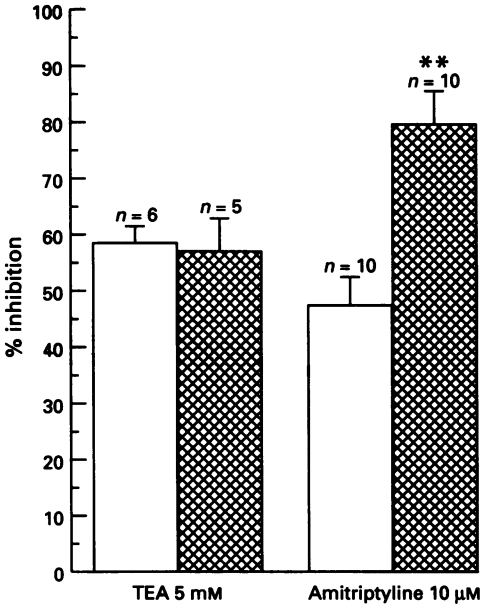


**Figure 7** Percentage inhibition of $K_V$ by 5 mM TEA and 10 $\mu$M amitriptyline. The inhibition of $K_V$ of amitriptyline is significantly greater ($P<0.05$) at pH 9.4 compared to pH 7.4. Currents were activated using voltage protocol (2).

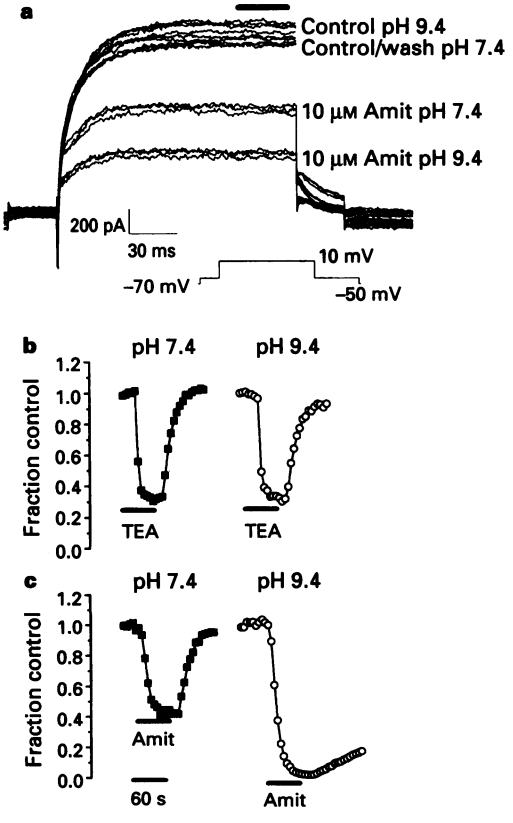


**Figure 6** The effects of 10 $\mu$M amitriptyline and 5 mM TEA on $K_V$ at pH 7.4 and 9.4 are shown. Currents in (a) were activated by voltage protocol (2). In (b) and (c) (■) represent data at pH 7.4 and (○) data at pH 9.4. In this cell 10 $\mu$M amitriptyline (Amit) inhibited $K_V$ by 59% at pH 7.4 and 95% at pH 9.4. TEA (5 mM) inhibited $K_V$ by 66% at both pHs. Note the slow wash of amitriptyline at pH 9.4.

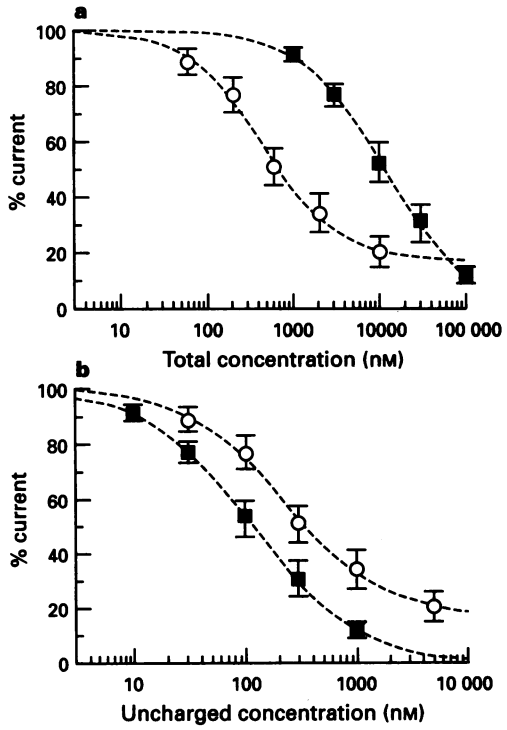


**Figure 8** Concentration-dependent inhibition of $K_V$ by amitriptyline at pH 7.4 (■) and pH 9.4 (○) are shown in (a). Data are fitted with equation [3]. (b) Shows the data from (a) plotted against uncharged drug concentration calculated from equation [4]. Currents were activated by voltage protocol (1).

between external uncharged drug concentration and potency of block.

Figure 8b shows the percentage inhibition of amitriptyline plotted as a function of uncharged concentration. If the external uncharged form of the drug was the only form which caused blockade of $K_V$ then the curves for data obtained at the two pHs would be superimposed in Figure 8b. In fact, there is some discrepancy between the two curves (a discrepancy also seen when comparing the effectiveness of the same uncharged concentration of tacrine at the two pHs, above). By taking the percentage inhibition as a function of the concentration of both the uncharged molecules and the charged molecules, the approximate contribution of each form can be calculated. Assuming that both species act at the same site but have different affinities, then at 50% inhibition the equation:

$$\{[U]/KU\} + \{[C]/KC\} = 1 \quad [5]$$

holds, where $KU$ and $KC$ are the respective equilibrium dissociation constants of the uncharged and charged forms (see Howe & Ritchie, 1991). By assuming that these are constant at the different pHs, simultaneous equations can be solved to reveal an approximate potency ratio between the two forms:

| | |
|---|---|
| At pH 7.4 | $119/KU + 11781/KC = 1$ |
| At pH 9.4 | $230/KU + 230/KC = 1$ |
| Thus | $KC/KU = 11551/111 = 103.4$ |

This would suggest that the uncharged form of amitriptyline is approximately 100 times more potent than the charged form.

### *Inhibition of $K_V$ by N-methylamitriptyline*

Figure 9 shows the effect of 10 $\mu$M concentrations of amitriptyline and its permanently charged analogue, N-methylamitriptyline on $K_V$. In 4 cells, amitriptyline caused an inhibition of 36.2±2.1% while in the same cells, N-methylamitriptyline was significantly less effective at blocking $K_V$, producing an inhibition of only 2.0±0.8%.

### *Effect of internal amitriptyline and N-methylamitriptyline*

The effect of amitriptyline on the intracellular face of the cell was investigated by adding 100 $\mu$M amitriptyline to the internal pipette solution. $K_V$ was measured at least 3 min after breakthrough to whole-cell recording configuration. There was no significant reduction in $K_V$ with amitriptyline applied intracellularly ($n = 11$ cells). It might be argued that internal amitriptyline is ineffective because, being lipophilic, it will

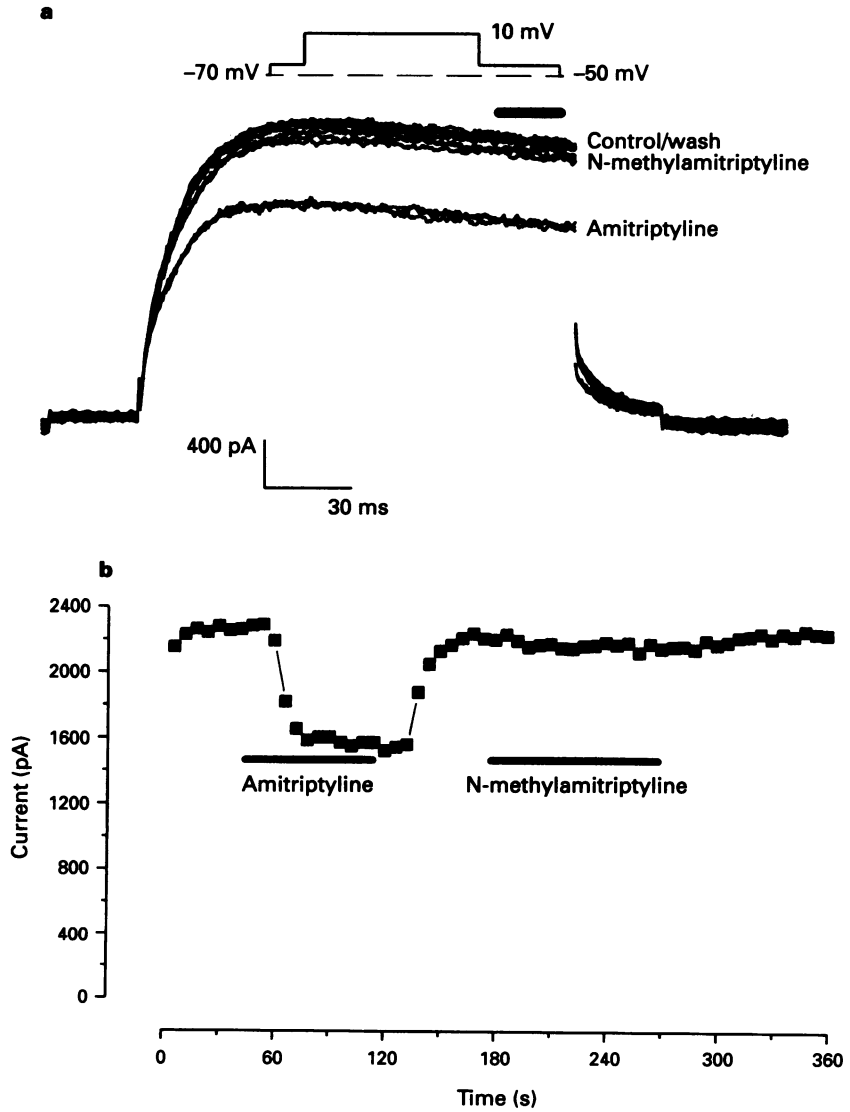


**Figure 9** The inhibition of $K_V$ by 10 $\mu$M amitriptyline and N-methylamitriptyline. Currents were activated by voltage protocol (2). For clarity only 12 traces are shown (3 in each condition) in (a). $K_V$ was measured as indicated by the bar in (a). The time course of drug action is shown in (b) where the bars indicate drug application.

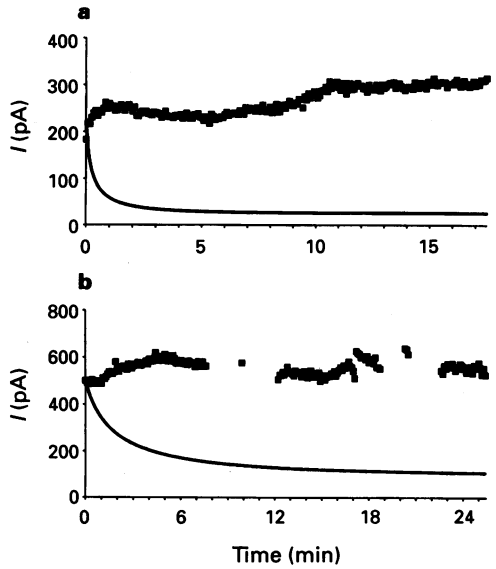


**Figure 10** The lack of effect of intracellular application of amitriptyline (100 $\mu$M) or N-methylamitriptyline (50 $\mu$M) on $K_V$. In (a) (■) shows the amplitude of $K_V$ evoked by voltage protocol (1) in a single cell at various time intervals over a period of 17 min following patch rupture. The solid line shows the predicted time course and degree of $K_V$ block if the amitriptyline acted from inside the cell to cause block (see Methods). For this cell, Rs = 11 MΩ, Cs = 10 pF and the calculated time constant for amitriptyline entry ($\tau_{ami}$) was 144 s. In (b) (■) shows the amplitude of $K_V$ evoked by voltage protocol (2) in a single-cell at various time intervals over a period of 25 min following patch rupture. The gaps between data points reflect periods of time where current-voltage relationships were measured or where the cell was held at −70 mV without stimulation. The solid line shows the predicted time course and degree of $K_V$ block if N-methylamitriptyline acted from inside the cell to cause block. For this cell, Rs = 8 MΩ, Cs = 29 pF and the calculated time constant for N-methylamitriptyline entry ($\tau_{amiM}$) was 573 s.

diffuse straight out of the cell from the pipette tip and so never reach the internal face of the majority of the channels in the cell. To address this point, similar experiments were carried out using the permanently charged analogue, N-methylamitriptyline, applied intracellularly. N-methylamitriptyline (50 $\mu$M), was also ineffective at reducing $K_V$ ($n = 6$) when applied intracellularly after at least 3 min of dialysis.

From the molecular weight of the drugs and the whole-cell capacitance and series resistance of these cells (see Methods and Figure 10 legend), 3 min is more than twice as long as that calculated for the intracellular concentration of either of these drugs to exceed 10 $\mu$M (see Pusch & Neher, 1988; Beech *et al.*, 1991; Stansfeld & Mathie, 1993). Nevertheless, currents were examined for as long a period of time as possible following breakthrough to whole-cell mode to determine whether any block developed slowly with time. This is illustrated in Figure 10a for amitriptyline and Figure 10b for N-methylamitriptyline. There was found to be no reduction in the current following internal dialysis with amitriptyline over a period of 17 min or N-methylamitriptyline over a period of 25 min. The lack of effect on $K_V$ can be compared with the solid line which shows the predicted time course and degree of inhibition of current in each of these cells if the charged form of amitriptyline acted from inside the cell to produce block (see Methods and Figure 10 legend).

Thus amitriptyline and N-methylamitriptyline appear to be ineffective at blocking $K_V$ when added to the inside of the cell.

## Discussion

In the first part of this study, we have shown that the tricyclic compounds cyproheptadine and dizocilpine can block potassium currents in rat sympathetic neurones. Cyproheptadine has long been known to possess potent 5-hydroxytryptamine and histamine antagonist properties. More recently, however, it has been suggested that the drug may act to block certain ion channels such as calcium channels in smooth muscle (Lowe *et al.*, 1981) and cardiac muscle (Riccioppo, 1983) and potassium channels in rabbit sinoatrial node (Kotake *et al.*, 1987). Dizocilpine is used experimentally as a potent non-competitive antagonist of NMDA receptors (e.g. Wong *et al.*, 1986; Halliwell *et al.*, 1989). It is however, not a selective blocking drug in that it blocks certain neuronal nicotinic receptors over a similar concentration range (1–10 $\mu$M) and at higher concentrations (~100 $\mu$M) blocks sodium channels (Halliwell *et al.*, 1989). The data in the present study show that a concentration of 10 $\mu$M (which is often the concentration that is used experimentally to block NMDA receptors) will have a significant action on neuronal potassium currents in the same cells. However, in contrast to actions on NMDA receptors both isomers of dizocilpine have equal potency on potassium currents.

The results with cyproheptadine and dizocilpine are consistent with our previous observations (Wooltorton & Mathie, 1993) that the potency of these tricyclic compounds in blocking $K_V$ is related to whether the molecule possesses a central three carbon chain separating the aromatic rings and the terminal tertiary amine (see Figure 1). Thus cyproheptadine, amitriptyline, chlorpromazine and imipramine are more effective blockers of $K_V$ than tacrine and dizocilpine.

Raising external pH caused a small but consistent increase in the amplitude of $K_A$ and $K_V$. This observation is consistent with previous work on the effects of pH on $K^+$ currents (e.g. Hille, 1973; Howe & Ritchie, 1991) and can be contrasted with the large effects seen on $Na^+$ current amplitude when varying external pH (see Hille, 1992). Changing pH did, however, alter the activation and inactivation voltage-range for the $K^+$ currents (see also, Howe & Ritchie, 1991) so that, for example, as pH was increased the $V_{50}$ for steady-state inactivation of both $K_V$ and $K_A$ moved to more negative potentials. For $K_V$, a 1 unit rise in pH caused a shift in the $V_{50}$ for inactivation of about 8 mV in the hyperpolarizing direction. Previously, this effect has been suggested to be due to hydrogen ions acting to neutralize negatively charged groups on the membrane surface (Howe & Ritchie, 1991) – i.e. a non-specific effect on membrane surface charge. However, the shifts in half inactivation potential observed are large in comparison with such small changes in external cation concentration, therefore, these effects may be better attributed to hydrogen ions binding to a particular receptive site on the channel protein to produce such effects on gating. A similar suggestion has been made for the large changes in the gating of transient potassium currents produced by low concentrations of certain divalent and trivalent cations such as zinc and lanthanum (e.g. Bardoni & Belluzzi, 1994; Watkins & Mathie, 1994; Talukder & Harrison, 1995).

Previously we have suggested that changing the external pH to alter the ratio of charged to uncharged tricyclic drug molecules may help to elucidate the mechanism by which these compounds produce their actions (Wooltorton & Mathie, 1993). It is clear from our results that raising the external pH enhanced the potency of these blocking drugs for inhibition of $K_V$ and this suggests that the uncharged form of the drug is required for potent block. This can be contrasted with muscarinic receptor binding by some of the same tricyclic compounds (amitriptyline and imipramine) where quaternary ammonium derivatives of the compounds were 2–4 fold more potent than the tertiary compounds (Ehlert *et al.*, 1990).

There are three possible explanations for the potency of the uncharged form. (1) The binding site on the channel for these compounds is extracellular and this site has a much higher affinity for uncharged molecules compared with charged. (2) The binding site is somewhere in the membrane and the uncharged form is required for the drug to enter the membrane. Block could then be caused, either by the uncharged form in

the membrane or the charged form gaining access to the inside of the channel. (3) The binding site (or access to it) is intracellular (for either charged or uncharged form) and the uncharged form is required for the drug to cross the membrane.

Our experiments where amitriptyline and N-methylamitriptyline were added to the internal solution and were ineffective would seem to provide strong evidence against proposal (3), namely, that the binding site for these compounds is intracellular. This can be contrasted with the mechanism of action of 4-aminopyridine, which is thought to block $K^+$ currents by binding to an intracellular binding site (e.g. Howe & Ritchie, 1991; Choquet & Korn, 1992; Stephens *et al.*, 1994). Experiments where 4-aminopyridine or its permanently charged analogue, 4-aminopyridine methiodide, were applied to the intracellular solution have shown clearly that these drugs act on an intracellular site of the potassium channel $mK_V$ 1.1 to cause block (Stephens *et al.*, 1994). This site is suggested to be at or near to the 'binding site' used by either the N-terminal ball peptide or the $\beta$ subunit of inactivating K channels to cause N-type inactivation (see e.g. Zagotta *et al.*, 1990; Pongs, 1992; Rettig *et al.*,1994). Furthermore, a similar mechanism and site of action has been proposed for a number of other drugs that block potassium channels, in particular, quaternary ammonium ions acting from inside the cell (e.g Armstrong, 1971; French & Shoukimas, 1981).

The major mechanism of action of local anaesthetic agents on sodium channels is also thought to involve the uncharged form of the molecule crossing the membrane, becoming charged and then reaching its binding site on the channel protein from the inside of the cell (e.g. Strichartz, 1973; Courtney, 1975; Narahashi & Frazier, 1975), although a different mechanism must apply to the neutral local anaesthetic agent, benzocaine (see Hille, 1992). From the results in this study, we must propose a different mechanism of action for block of $K_V$ by the tricyclic compounds tested. The characteristics of block show a number of similarities with block of $I_{K(f)}$ in rat melanotrophs by quinidine (Kehl, 1991) although quinidine increased the rate of decay of $I_{K(f)}$; an effect increased by membrane depolarization. Block by quinidine did not seem to be due to the drug acting from inside the cell. Furthermore, block by quinidine was neither voltage- nor use-dependent which is similar to what we have previously reported for tricyclic block of $K_V$ (Wooltorton & Mathie, 1993).

The simplest conclusion drawn from the results with quinidine was that the quinidine binding site was on the external face of the membrane (Kehl, 1991) similar to proposal (1) above. Our observation that the charged form of amitriptyline has a finite blocking action; although calculated as being around 100 fold less potent than uncharged amitriptyline; provides some support for proposal (1), namely an external binding site with a high affinity for the uncharged form and suggests proposal (2) is less likely to be correct.

Because the uncharged form of these compounds confers potency and this form exists at low concentrations around neutral pH, the effectiveness of these compounds in inhibiting $K_V$ will be increased if extracellular pH becomes alkaline. It can be calculated that the uncharged concentration will be approximately doubled for 0.3 of a pH unit rise in extracellular pH. It is noteworthy that activation of $GABA_A$, AMPA/kainate and NMDA receptors can give rise to extracellular alkaline shifts (Chesler & Kaila, 1992). These can be measured with pH microelectrodes as being 0.1 to 0.2 of a pH unit in size and this recorded response is thought to be only a fraction of the actual synaptic pH changes that occur due to the limited temporal and spatial resolution of such measurements (Chesler & Kaila, 1992).

In addition to the effects described here, it has been shown recently that a number of the same tricyclic compounds are potent blockers of calcium-activated $K^+$ currents in rat cortical neurones (Lee *et al.*,1995). However, none of the compounds used is a selective blocker of potassium currents (see above and Wooltorton & Mathie, 1993), indeed all of them are used primarily in clinical or experimental situations for other pharmacological actions which they possess. Despite this, block of $K^+$ currents will be evident at therapeutic concentrations of many of these tricyclic compounds (see Benet & Williams, 1990) and will be a major consideration at higher concentrations. Block of $K^+$ currents will prolong action potentials in both neuronal and cardiac tissue (Robbins & Sim, 1990; Zhang *et al.*, 1992) and this could easily account for the convulsive and dysrythmic adverse actions of tricyclic compounds when they are taken in overdose.

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